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Differentiation-Induced and Constitutive Transcription of Human Papillomavirus Type 31b in Cell Lines Containing Viral Episomes

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The expression of viral genes during the productive life cycle of human papillomaviruses (HPV) is tightly coupled to the differentiation program of epithelial cells. We have examined transcription of HPV as a function of differentiation in an in vitro organotypic raft culture system which allows for epithelial stratification at the air-liquid interface. When CIN612 cells, which contain episomal copies of HPV type 31b (HPV31b), were allowed to stratify in raft cultures, they differentiated in a manner which was histologically similar to that seen in a cervical intraepithelial neoplasia I biopsy lesion. In monolayer cultures of CIN612 cells, two major polycistronic HPV31b transcripts of 1.7 kb which encode (i) E6, E7, E1^E4, and E5 and (ii) E6*, E7, E1^E4, and E5 were identified. These RNAs initiated at a promoter, P97, in the upstream regulatory region of the virus. Following differentiation in raft cultures, the relative abundance of RNAs initiated at P97 was unchanged. In contrast, the expression of a 1.3-kb RNA encoding an E1^E4 fusion protein and E5 was found to increase substantially following differentiation. This transcript was initiated at a novel promoter within the E7 gene (P742). These studies have therefore identified a constitutive viral promoter which is active throughout stratified epithelium as well as a novel promoter which is induced upon epithelial cell differentiation.

Papillomaviruses are epitheliotropic viruses which induce a variety of proliferative lesions in mammals. Over 60 types of human papillomaviruses (HPV) have been identified, and these show a preference for either mucosal or cutaneous tissue (16a). Of the strains that infect the genital mucosa, certain types, notably HPV type 16 (HPV16), HPV18, and HPV31 (high-risk types), are associated with increased risk for cervical cancer, while others, such as HPV6 and HPV11 (low-risk types), are rarely associated with neoplasia (8, 19, 35, 37, 60). Immortalized cell lines can be established by transfection of primary cells with DNA from the high-risk types or from biopsies of lesions infected with these types but not from low-risk strains (27, 36, 53, 58). Thus, ability to transform cells in vitro correlates with oncogenic potential.

HPV show a high degree of conservation with respect to genome structure and organization. The viral genome is approximately 7.9 kb in size and consists of eight open reading frames (ORFs) which are all transcribed from the same strand (reviewed in reference 9). Expression of early genes is directed by the upstream regulatory region, which contains promoter and enhancer elements. Two HPV ORFs, E6 and E7, are selectively retained and expressed in carcinoma cell lines and are capable of immortalizing keratinocytes in vitro (3, 4, 6, 25, 42–44). The E6 and E7 gene products bind to p53 and the retinoblastoma gene product, respectively (20, 57). The E2 gene product is a transcriptional transactivator which interacts with sequences in the upstream regulatory region (1, 15, 24, 50), while the E1 protein is thought to play a role in replication of the viral DNA (56). The late gene products, L1 and L2, are the viral capsid proteins.

Papillomaviruses infect squamous epithelial cells and alter their normal differentiation. In low-grade HPV-induced lesions, the basal layer, which normally consists of one to two layers of mitotically active cells, occupies up to one-third of the epithelium. The remainder of the epithelium undergoes a slightly altered differentiation program, with virion production occurring in the highly differentiated suprabasal cells. In infections of the cervix, these low-grade lesions are called condyloma or cervical intraepithelial neoplasia (CIN) I (30) and can progress to higher-grade CIN II and CIN III lesions (59, 60). In CIN III lesions, the ability to differentiate is lost completely and basal-like cells occupy the entire thickness of the epithelium. No virus is produced in high-grade CIN III or carcinoma in situ.

The viral life cycle is closely linked to the differentiation program of the stratifying epithelium (16, 51, 52). The virus infects basal cells, in which viral genomes are established as episomes and viral gene expression is limited to the early region. In contrast, expression of the late genes and viral DNA amplification is restricted to the upper, more differentiated layers of the epithelium. Most in vitro studies examining HPV transcription have been limited to monolayer cultures (3, 42–44, 49), which resemble undifferentiated basal cells with respect to their expression of both viral and cellular genes. It has therefore been difficult to study viral gene expression of the high-risk viruses during productive infection. The transcripts expressed by the low-risk HPV11 strain in biopsies of condyloma and xenograft cultures have been studied by polymerase chain reaction (PCR) and electron microscopic analysis of RNA (14, 40). The HPV11 early genes were found to be expressed either from promoters located upstream of the E6 ORF or from the end of the E7 gene. These RNAs were found to be differentially spliced, multicistronic transcripts which were terminated at a single polyadenylation site at the 3′ end of the early region. While these analyses permit a fairly detailed characterization of the RNAs encoded by low-risk strains, they have not identified changes in HPV transcription that occur as a function of

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differentiation. Such studies have been largely limited to in situ hybridization of biopsy material (16, 51, 52). These studies have shown a constant level of E6/E7 expression throughout the stratified epithelium. In contrast, a dramatic increase in expression of E4, as well as of L1/L2, was observed in suprabasal cells. While in situ hybridization permits a general characterization of transcribed regions, it does not allow for the identification of particular transcripts. Thus, the study of differentiation-specific transcripts and their corresponding promoters remains incomplete.

We have derived a cell line, CIN612, established from a biopsy of a patient with a CIN I lesion (5, 38). This cell line is unusual in that it maintains HPV31b genomes as episomes, whereas HPV DNA is found integrated in cell lines established from cervical carcinomas or from transfected keratinocytes. Integration of the viral genome usually occurs within E1 or E2 and thus disrupts the normal pattern of viral transcription. In contrast to cells derived from carcinomas, early passages of CIN612 cells retain the ability to differentiate when grown in vitro on collagen plugs at an air-liquid interface (raft cultures) (5). We have used CIN612 cells grown on monolayers and on collagen rafts to characterize the changes in HPV gene expression that occur as a function of epithelial differentiation in vitro. Our results show that there is a substantial increase in expression of RNA encoding the E4 and E5 ORFs as a function of differentiation in vitro and that this is due to an increase in the abundance of a transcript initiated from a promoter in the E7 ORF.

MATERIALS AND METHODS

Cell culture. The CIN612 cell line was derived from a biopsy specimen of a patient with a CIN I lesion (38) and maintained in E medium with mitomycin-treated fibroblast feeders (34). Subclones were isolated by limiting dilution of the parent line and expansion of single colonies (5). Collagen raft cultures for in vitro differentiation were prepared as described previously (2, 29, 34). Cells were seeded onto a collagen plug containing fibroblast feeders and grown to confluence. The plugs were then lifted onto a stainless steel grid, and the epithelial cells were allowed to stratify and differentiate at the air-liquid interface for 2 weeks.

DNA analysis. Total cell DNA was isolated from trypsinized cells by proteinase K digestion and phenol extraction as previously described (7). DNAs were digested with restriction enzymes according to the directions of the manufacturer, electrophoretically separated on 0.8% gels, irradiated for 10 min to nick the DNA, denatured, neutralized, and transferred to a nylon membrane (MSI, Westboro, Mass.). Blots were hybridized with gel-purified HPV31 DNA labeled by random priming (Boehringer Mannheim random priming kit) at 42°C in 50% formamide–10% dextran sulfate–100 μg of denatured salmon sperm DNA per ml–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% sodium dodecyl sulfate (SDS)–0.02% polyvinylpyrrolidone–0.02% Ficoll–20 mM sodium phosphate (pH 7.0).

Cloning of episomal HPV31b DNA. Hirt extracts for isolation of episomal DNA were prepared from lysates of 612-5 cells as previously described (13). Hirt DNA was digested with *Xba*I to linearize HPV DNA and with *Bam*HI, a noncutter for HPV31, to reduce background in cloning. Hirt DNA was electrophoretically separated on a low-melting-point agarose gel, and DNA fragments in the 8-kb region were excised and extracted from the gel. HPV31b DNA was cloned into the *Xba*I site of λZap II (Stratagene). Recombinant phage containing HPV31b DNA were identified by in situ hybridization with ^{32}P-labeled HPV31 DNA (32) and plaque purified. Recombinant phage DNA was purified from large-scale cultures, and HPV31b was subcloned into the *Xba*I site of Bluescript (Stratagene).

RNA isolation and Northern (RNA) blot analysis. Total cell RNA was isolated from submerged cultures by the guanidinium isothiocyanate-CsCl centrifugation method (11). Polyadenylated RNA was selected by oligod(T)-cellulose chromatography. For analysis of RNA from differentiated cells, cells from the 9E subclone of CIN612 were grown on 5-cm-diameter rafts. After 2 weeks, the cell layer was scraped off the collagen plug with a scalpel and dissolved in 50 ml of guanidinium isothiocyanate. Approximately 5 mg of total cell RNA was recovered after centrifugation through a 5.7 M CsCl cushion. For Northern blot analysis, 5 μg of polyadenylated or total cell RNA was electrophoretically separated on 0.8% agarose–2.2 M formaldehyde gels and transferred to MSI paper. For high-stringency hybridizations, blots were hybridized for 18 h at 42°C in 50% formamide–10% dextran sulfate–100 μg of denatured salmon sperm DNA per ml–5× SSC–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES, pH 6.5)–0.2% SDS–0.02% polyvinylpyrrolidone–0.02% Ficoll and washed at 65°C in successively lower salt concentrations, with a final wash of 0.1× SSC–0.2% SDS. For low-stringency hybridizations, blots were hybridized at 55°C without formamide, and the final wash was done at 55°C in 1.3× SSC–0.2% SDS.

Probes specific for different ORFs were amplified by PCR (26), gel purified, and labeled with a random priming kit (Boehringer Mannheim). The following nucleotide sequences were used for probes: E6/E7, 108 to 868; E6, 108 to 462; E7, 541 to 877; 5′ E7, 541 to 780; 3′ E7, 763 to 877; E1, 862 to 2775; E2, 2764 to 3264; E4/E5, 3311 to 4050; and E4, 3311 to 3517. Nucleotide positions are assigned from the HPV31 genome (22). The E6 intron (nucleotides [nt] 211 to 412) was amplified by PCR and cloned into the *Sma*I site of Bluescript, excised from the plasmid by using restriction sites in the polylinker, and gel purified. Results were quantitated on a Betascope 603 blot analyzer (Betagen, Waltham, Mass.).

cDNA isolation. cDNAs were synthesized from 5 μg of polyadenylated 612-5 RNA by using a cDNA synthesis kit (Pharmacia) and oligo(dT)$_{12-18}$ as a primer. The blunt-end cDNA was ligated first to an adaptor containing an *Eco*RI overhang and an internal *Not*I site and then to λgt11 DNA which had been digested with *Eco*RI and treated with phosphatase (Stratagene). The ligated DNA was packaged in vitro (Stratagene Gigapack II Gold) and plated on Y1090r$^-$ cells at a density of 5×10^4 PFU per plate. Positive plaques were identified by in situ hybridization using gel-purified HPV31b DNA as a probe and plaque purified. Eleven cDNA clones were isolated from a library of $\sim 8 \times 10^5$ PFU. cDNAs were amplified by PCR from purified plaques by using oligonucleotides flanking the *Eco*RI sites in recombinant phage. The PCR products were either made blunt end and cloned into the *Sma*I site of Bluescript or digested at the *Not*I site in the adaptors and cloned into the *Not*I site of Bluescript. Double-stranded cDNA plasmids were sequenced with Sequenase 2.0 (U.S. Biochemical).

Primer extension and RNase protection analyses. Primer extension analysis was performed with a 42-base oligonucleotide complementary to nt 421 to 462 according to published procedures (28). Briefly, 5×10^4 cpm of end-labeled primer was hybridized overnight to 10 μg of polyadenylated RNA at 30°C in 80% formamide–40 mM PIPES (pH 6.5)–0.4 M NaCl–1 mM EDTA, ethanol precipitated, and extended at

42°C for 90 min with moloney murine leukemia virus reverse transcriptase. The products were phenol extracted, ethanol precipitated, and analyzed on 6% acrylamide–7 M urea gels. The sizes of the primer extension products were determined from sequencing ladders and from end-labeled *Hpa*II restriction fragments of Bluescript.

RNase protection assays (21) were performed with several regions of the HPV31b genome, which were amplified by PCR and subcloned into Bluescript. For analysis of the E6 intron and flanking sequences, a *Pst*I-*Hin*fI PCR fragment (nt 121 to 478) was made blunt end with T4 DNA polymerase and cloned into the *Sma*I site of Bluescript. An *Acc*I fragment (nt 200 to 747) was cloned into the Bluescript *Acc*I site for analysis of the 5′ ends of E6/E7 RNAs. To analyze transcripts containing the E4 splice junction, an *Rsa*I-*Hin*cII fragment (nt 3172 to 3503) was cloned into the *Sma*I site of Bluescript. A *Pvu*II-*Hin*cII fragment (nt 684 to 1006) was cloned into the *Sma*I site of Bluescript to analyze RNAs containing the E1 splice junction. A *Pvu*II-*Hin*cII fragment from a cDNA encoding E6*, E7, E1^E4, and E5 was subcloned into the *Sma*I site of Bluescript to analyze transcripts containing the E4 ORF. This clone contains nt 684 (*Pvu*II site) through 877 (E1 splice junction) joined to a segment containing nt 3295 (E4 splice junction) through 3503 (*Hin*cII site). All clones were sequenced to determine orientation, and an antisense probe was synthesized in vitro, using a Stratagene in vitro transcription kit and T3 RNA polymerase. Probes were gel purified, and approximately 5 × 10^5 cpm was hybridized overnight to 10 μg of total cell RNA at 42°C in 80% formamide–0.4 M NaCl–40 mM PIPES (pH 6.5)–1 mM EDTA after denaturation at 85°C for 5 min. After ethanol precipitation and digestion with RNases, the protected fragments were purified by proteinase K digestion and phenol extraction, ethanol precipitated, and analyzed on 6% acrylamide–7 M urea gels, using end-labeled *Hpa*II DNA fragments of Bluescript as size markers. The results were quantitated on a Betascope 603 blot analyzer (Betagen) or by densitometric analysis of the autoradiogram with an LKB Ultroscan XL densitometer.

RESULTS

We have examined the transcription patterns of HPV31b in several subclones of CIN612 which contain various forms of viral DNA. A subset of clonal cell lines contain only episomal copies of HPV DNA. Previous analysis using two-dimensional gel electrophoresis has confirmed the predominance of episomal forms in these cells (5). Others contain only integrated DNA, and some contain both. As the cells are passaged, cells containing integrated copies of HPV DNA tend to predominate the culture, presumably because they have a growth advantage. Figure 1 shows a Southern blot analysis of the DNAs of five subclones, 7E, 9E, 612A, 612-5, and 612-6. While 7E and 9E contain primarily episomal copies of HPV31b, 612-5 was found to contain both integrated and episomal DNA. The 612-6 cell line contains only integrated DNA, which is approximately single copy.

Analysis of the HPV31b transcripts produced in monolayer cells. We characterized the viral transcripts present in monolayer cultures of three subclones of CIN612 cells, 7E, 9E, and 612-5, by Northern blot hybridization with subgenomic probes specific for different ORFs (Fig. 2). Lanes 1 and 2 of each panel show hybridization to RNAs from 7E and 9E, which contain primarily episomal DNA. A probe specific to the E6/E7 region hybridized predominantly to a 1.7-kb RNA and to much less abundant transcripts of 4.3 and 2.3 kb in 7E and 9E cells (Fig. 2A, lanes 1 and 2). The 1.7-kb RNA also hybridized to probes from the E4/E5 region. The 4.3-kb RNA hybridized to probes for E1, E2, and E4/E5 and is likely to encode the entire early region. In contrast, the 2.3-kb RNA hybridized to probes from E2 and E4/E5 (Fig. 2B and C, lanes 1 and 2). A 1.3-kb RNA which was specific to the E4/E5 ORF was also observed (Fig. 2C). To identify

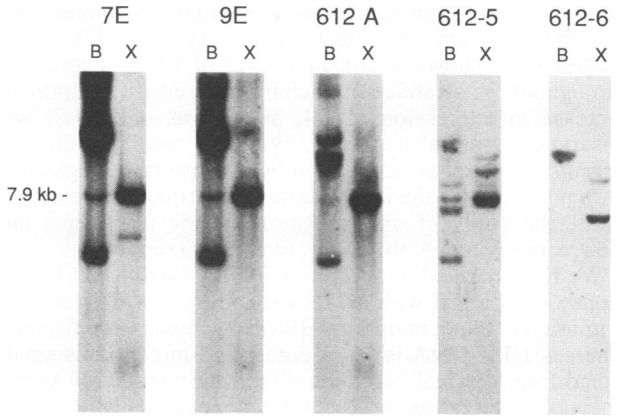

FIG. 1. Southern blot of DNAs of CIN612 subclones. DNAs from subclones 7E, 9E, 612A, 612-5, and 612-6 were digested with *Bam*HI (lanes B) or *Xba*I (lanes X), separated on 0.8% agarose gels, transferred to a nylon membrane, and hybridized to an HPV31b probe. Digestion with *Bam*HI, which has no recognition sites within the HPV31b genome, reveals a rapidly migrating species characteristic of episomal DNA in 7E, 9E, 612A, and 612-5. The high-molecular-weight bands present in the *Bam*HI digests of 7E, 9E, and 612A are concatenates of episomal DNA. Also present is some linear DNA which may have been generated by nicking during isolation. Some integrated DNA, which is smaller than 7.9 kb and must therefore be partially deleted, is also detectable in the *Xba*I digest of 7E DNA. The additional bands present in the *Bam*HI and *Xba*I digests of 612-5 DNA indicate that this cell line contains integrated copies of HPV31b in addition to episomes. The exposure time for 612-6 is four times greater than for the other subclones.

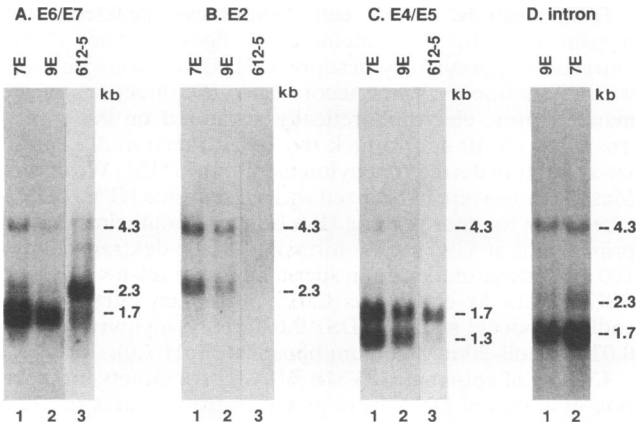

FIG. 2. Northern blot analysis of RNAs expressed in subclones of CIN612 cells. Polyadenylated (A to C) and total cell (D) RNAs were separated on 0.8% agarose–2.2 M formaldehyde gels, transferred to a nylon membrane, and hybridized to probes specific for the E6 and E7 ORFs (A), the E2 ORF (B), E4 and E5 ORFs (C), or the E6 intron (D). Hybridization to the 4.3-kb RNA is detectable with all probes from the early region upon longer exposure. Exposure times were varied to optimize visualization of the bands.

transcripts likely to encode a full-length E1 ORF, we used an E1-specific probe, which was found to hybridize only to the 4.3-kb RNA (data not shown).

We also examined the HPV transcripts of 612-5 cells, which contain both integrated and episomal copies of HPV DNA (Fig. 1). The 1.7-kb RNA, which was the predominant E6/E7 RNA present in episomal cell lines, was also present in 612-5 cells (Fig. 2A). The most abundant transcript encoding E6 and E7 in 612-5 cells was 2.3 kb. Several higher-molecular-weight transcripts were also observed. Unlike the E6/E7 RNAs detected in other cell lines, these transcripts were specific to the E6/E7 region and are likely derived from integrated copies of the DNA (see below). The 1.3-kb RNA encoding E4 and E5 was much less abundant in 612-5 cells but was detectable upon longer exposure. In contrast to cell lines which contain predominantly episomal copies of DNA, transcripts encoding E2 were not detectable in 612-5 cells.

Transcripts from high-risk HPV strains often encode a truncated form of E6, designated E6*, which is generated by removal of an intron in the E6 gene (44). These are much more abundant than those encoding a full-length E6 in many cell lines containing HPV16 or HPV18 (18, 44, 49). To identify HPV31b RNAs encoding a full-length E6, we made a probe specific for the intron in the E6 ORF. Using this probe, we detected hybridization to RNAs of 4.3, 2.3, and 1.7 kb in cell lines containing episomal copies of HPV31b (Fig. 2D). Thus, significant levels of RNAs encoding full-length E6 are expressed in CIN612 cells.

Mapping the 5′ ends of E6/E7 transcripts. We next used primer extension and RNase protection analysis to map the 5′ ends of the transcripts present in monolayer cultures (Fig. 3). For primer extension (Fig. 3A), we used a primer with a 5′ end at nt 462, which is downstream of the intron in the E6 ORF (Fig. 3C). Two primer extension products were observed in 612-6 and 612A cells as well as 7E, 9E, and 612-5 cells. The first is a weak band at 365 bases which most likely came from an unspliced transcript initiated at nt 97. The second is a much more prominent species of 163 bases. This species is consistent in size with an E6* transcript which is initiated at P97 and from which the 201-base intron in the E6 ORF has been removed. P97 also serves as the promoter for initiation of early-region transcripts in other HPV strains (42, 49).

In addition to the promoter at nt 97 which is used to express E6, E6*, and E7, other investigators have shown that low-risk HPV types utilize a separate promoter within the E6 ORF for transcription of E7 (48). We used RNase protection assay with an antisense RNA probe containing nt 200 to 747 in order to detect HPV31b transcripts initiated in E6. The results (Fig. 3B) show one 547-base fragment corresponding to the full length of HPV sequences in the probe. This fragment is derived from unspliced RNA encoding E6. A second, more abundant protected fragment of 334 bases, the size expected from an E6* transcript spliced at nt 413 and extending through the E7 ORF, was also detected. No smaller protected fragments, which would be expected from transcripts initiated in the E6 ORF, were observed. Thus, the only detectable promoter for initiation of RNAs encoding E6, E6*, and E7 in the CIN612 cell line is at nt 97. The RNase protection assay, which uses a uniformly labeled probe and permits direct comparison of the products of both E6 and E6*, affords the best comparison of the relative abundance of E6 and E6*. On the basis of densitometric analysis, E6* transcripts are on average approximately

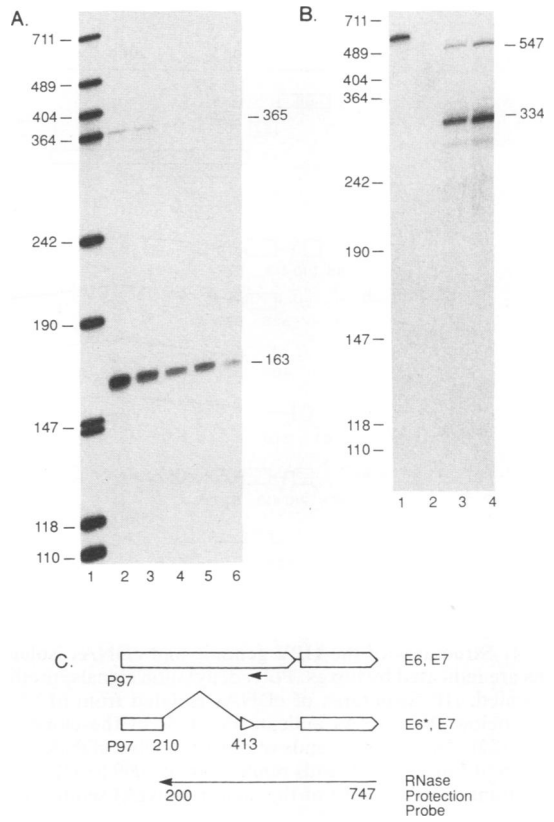

FIG. 3. Identification of 5′ ends of transcripts encoding E6 and E7. (A) Primer extension analysis. Five micrograms of polyadenylated RNA from subclones of the CIN612 cell line was annealed to a primer end labeled at nt 462 and extended with reverse transcriptase. Lanes: 1, end-labeled *Hpa*II fragments of Bluescript; 2 to 6, primer extension products of RNA from 7E, 9E, 612A, 612-5, and 612-6 cells, respectively. (B) RNase protection assay with an antisense RNA probe spanning nt 747 to 200. Lanes: 1, RNA probe; 2, products protected by tRNA; 3 and 4, products protected by RNA from 9E and 7E cells, respectively. Numbers at the left indicate the positions of migration (indicated in bases) of Bluescript *Hpa*II DNA fragments used as size markers; numbers at the right indicate sizes (in bases) of protected fragments. The 547-base fragment arises from unspliced transcripts which protect all of the HPV sequences in the probe. This fragment is smaller than the probe, which also contains 37 bases of plasmid sequences. The 334-base fragment is consistent in size with an E6* transcript initiated at P97. No additional fragments indicative of an E7-specific promoter were detected. (C) Schematics of transcripts encoding E6, E6*, and E7 and of the RNase protection probe. Numbers below the E6*,E7 transcript indicate coordinates of splice junctions. Boxes indicate ORFs. The small arrow indicates the position of the primer used for panel A.

seven times more abundant than those encoding full-length E6 in these cell lines.

cDNA analysis of HPV transcripts. To characterize the RNAs encoded by HPV31b in detail, we constructed a cDNA library from 612-5 RNA, which has transcripts from both integrated and episomal copies of HPV. Eleven cDNAs were isolated and sequenced (Fig. 4). Five of these clones were very similar in structure and were most likely derived from the same transcript (Fig. 4B, sequence a). These cDNAs were derived from multicistronic mRNAs containing a spliced E6 ORF, E7, 16 bases of the E1 ORF joined to E4, and the E5 ORF. The splice junction within the E6 ORF

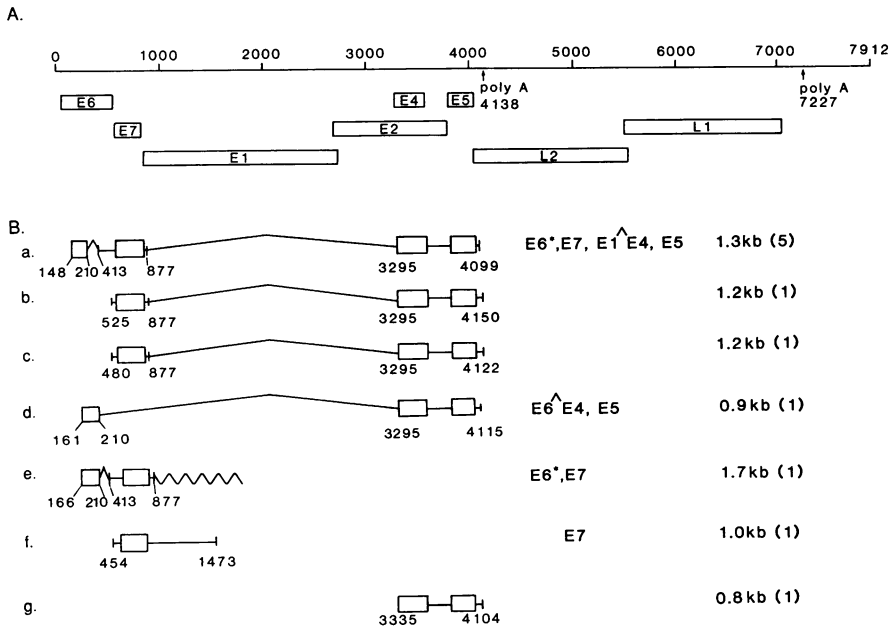

FIG. 4. Structures of the HPV genome and cDNAs isolated from 612-5 cells. (A) Structure of the HPV genome. ORFs encoding HPV proteins are indicated by boxes. Polyadenylation signals, predicted from the HPV31 sequence, for the early (nt 4138) and late (nt 7227) regions are indicated. (B) Structures of cDNAs isolated from 612-5 cell RNA. Numbers in parentheses indicate the numbers of clones isolated; numbers below the cDNAs indicate the ends of the clones and splice junctions. Nucleotide coordinates are based on the HPV31 DNA sequence (22). The 5′ and 3′ ends of one of the five cDNAs encoding E6*, E7, E1^E4, and E5 are indicated. The 5′ ends of the other cDNAs range from nt 136 to 171; 3′ ends range from nt 4099 to 4125. Sizes of the cDNAs are shown in kilobases. These cDNAs are somewhat smaller than the transcripts because of the lack of poly(A) sequences and some 5′ sequence.

joins base 210 to base 413, generating E6*, a 36-amino-acid protein which terminates immediately downstream of the splice junction (Fig. 5). E6* has been previously detected in RNAs of other high-risk HPV strains, but the low-risk strains lack these splice sites. The splice which joins base 877 in the E1 ORF to base 3295 in the E4 ORF (Fig. 5) generates an E1^E4 fusion protein containing only five amino acids from E1. This fusion protein retains the ATG at the beginning of the E1 ORF and so provides a means for translation of the E4 ORF, which lacks its own ATG. Since the 5′ ends of the cDNAs range from nt 136 to 171 and the major promoter that we have mapped is at nt 97, these

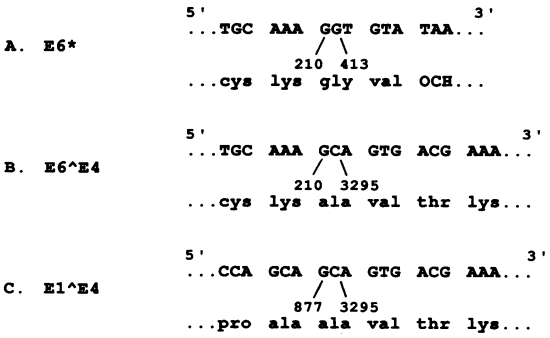

FIG. 5. Nucleotide sequences of splice junctions of cDNA clones. (A) Splice junction in E6 which joins nt 210 to nt 413 to generate E6*. (B) Splice junction which joins the donor site in E6 (nt 210) to an acceptor in E4 (nt 3295) to generate an E6^E4 fusion protein. (C) Splice junction joining nt 877 in E1 to the acceptor in E4 at nt 3295, which generates an E1^E4 fusion protein.

cDNAs are likely nearly full-length copies of the RNA. The 3′ ends of these cDNAs range from 4099 to 4125, which is very close to the polyadenylation site at 4138 that is predicted from the HPV31 DNA sequence (22) and from RNA analysis of other HPV strains (14). Since the cDNAs that we isolated were not polyadenylated, they are smaller than their actual size in vivo. They are therefore likely copies of the 1.7-kb RNA detected on Northern blots with probes from E6/E7 and E4/E5. Two other cDNAs contain the E7, E1^E4, and E5 ORFs but terminate in E6 just upstream of the E7 ORF. Since we do not detect transcripts initiated in E6 by RNase protection assay (Fig. 3B), these are most likely partial copies of the 1.7-kb RNA.

A novel 0.9-kb cDNA was detected in which the splice donor at nt 210 in E6 is joined to the acceptor at nt 3295 in E4, skipping over the exon encoding E7 (Fig. 4B, sequence d). This transcript would be approximately 1.3 kb in length and has the potential to encode an E6^E4 fusion protein in addition to E5 (Fig. 5B). A similar transcript has been reported in the W12 cell line, which contains episomal copies of HPV16, but this transcript encodes E6* rather than an E6^E4 fusion protein (18). The transcript encoding E6^E4 is approximately 1.3 kb in size. A second novel 1.7-kb cDNA containing most of the spliced E6 ORF, E7, and E1 spliced to unknown sequences (Fig. 4B, sequence e) was observed in 612-5 cells. This transcript is similar to viral/cellular chimeric transcripts which have been described in carcinoma cell lines (44). A search of the GenBank data base did not reveal significant homology to any known sequences, and a probe specific for these sequences did not hybridize to any RNAs present in other subclones of CIN612 cells. We believe that this cDNA is a copy of the abundant 2.3-kb RNA detected in 612-5 cells with E6/E7 probe and is derived

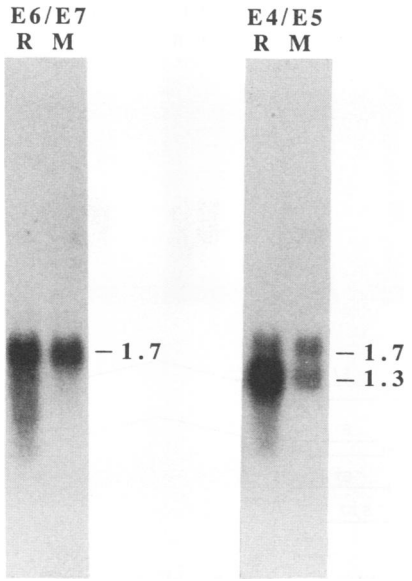

FIG. 6. Comparison of HPV31b transcripts present in differentiated and undifferentiated keratinocytes by Northern blot analysis. Five micrograms of 9E total cell RNA was isolated from monolayer (lanes M) or raft (lanes R) cultures, separated on 0.8% agarose–2.2 M formaldehyde gels, transferred to a nylon membrane, and hybridized to probes specific for the E6/E7 or E4/E5 ORF. Sizes are indicated in kilobases.

from an integrated copy of HPV DNA. In addition, we isolated two other cDNAs: a 1.0-kb cDNA encoding only the E7 ORF (Fig. 4B, sequence f) and a 0.8-kb cDNA which contains most of the E4 ORF and E5 and terminates near the early polyadenylation site (Fig. 4B, sequence g). We believe these to be partial copies of larger RNAs. Although we detected transcripts encoding full-length E6 by Northern blotting and RNase protection, we did not isolate any cDNAs encoding these genes. We therefore believe that our cDNA library is not exhaustive.

Comparison of HPV RNAs expressed in differentiated and undifferentiated keratinocytes. The transcripts described above were isolated from monolayer cultures, which may not reflect expression in stratified epithelial cells. When cells are grown on collagen rafts at the air-liquid interface, they stratify and differentiate in a manner which closely resembles that found in histological cross-sections of CIN I lesions in vivo (38). Previous studies have shown a differentiation-dependent induction of late viral gene expression in CIN612 cells grown in raft cultures (5). We further characterized the effect of cellular differentiation on viral gene expression by comparing RNAs from CIN612 9E cells grown in monolayers and on rafts. Northern blot analysis (Fig. 6) showed that in rafts and in monolayers, the most abundant transcript encoding E6 and E7 was 1.7 kb. An E4/E5 probe was found to hybridize predominantly to RNAs of 1.7 and 1.3 kb in both monolayer and raft cultures. While the levels of these transcripts were similar in monolayer cells, the 1.3-kb RNA was much more abundant in cells grown on rafts. No change in the level of expression of E1 and E2 was observed, but low levels of late-region expression were observed specifically in raft cultures (data not shown).

On the basis of the cDNAs isolated from 612-5 cells, it seemed possible that the 1.3-kb RNA could encode the E6^E4 fusion protein as well as E5. The increase in abundance of this transcript would then be due to preferential usage of a particular splicing pattern upon differentiation. We first confirmed that the E6^E4 transcript was expressed in 9E cells by sequencing the PCR product generated from the RNA, using primers from the E6 and E4 ORFS (data not shown). RNase protection (Fig. 7) was used to demonstrate that there was no change in the frequency of splice site usage in raft and monolayer cultures. Thus, the 1.3-kb RNA which increases in abundance in differentiated keratinocytes is unlikely to encode the E6^E4 protein.

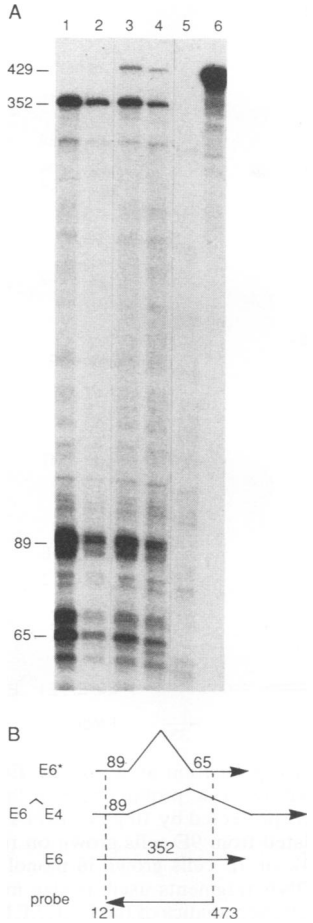

FIG. 7. Comparison of E6 transcripts present in differentiated and undifferentiated keratinocytes by RNase protection assay. (A) RNase protection of a probe spanning the E6 intron with 1.0 or 0.5 μg of total cell RNA from monolayer (lanes 1 and 2) or raft (lanes 3 and 4) cultures or with 10 μg of tRNA (lane 5). Lane 6, undigested probe. Numbers at the left indicated sizes (in bases) of protected products. The undigested probe is 77 bases larger than the fully protected probe because of the presence of Bluescript sequences. The 352-base fragment is protected by unspliced RNAs encoding E6. The 89-base fragment is protected by RNAs encoding both E6*,E7,E1^E4,E5 and E6^E4, while the 65-base product is protected only by the E6*,E7,E1^E4,E5 transcript. There is no change in the relative abundance of these fragments in RNAs from raft and monolayer cultures. The abundance of the E6^E4 RNA therefore does not change upon differentiation. (B) Schematics of transcripts encoding E6*, E6^E4, and full-length E6, showing expected protected regions.

Mapping the 5′ end of the E4/E5 transcript. Since the E4 ORF has no initiating ATG, the 5′ end of the 1.3-kb E4/E5 transcript which increases in abundance in raft cultures

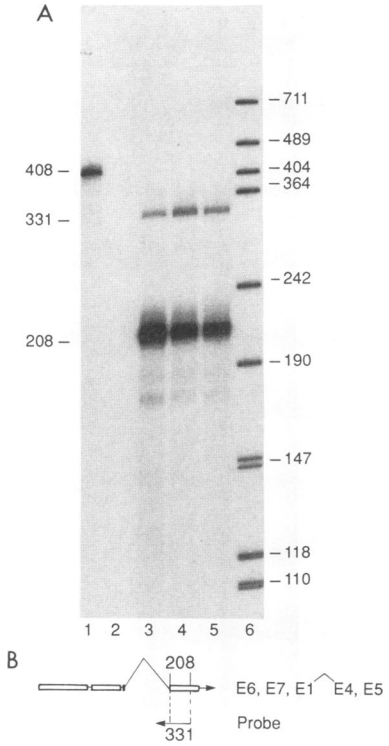

FIG. 8. (A) RNase protection assay of the E4 splice junction. Lanes: 1, undigested 408-base probe spanning the E4 splice junction; 2 to 5, products protected by 10 μg of tRNA (lane 2), 10 μg of total cell RNA isolated from 9E cells grown on rafts (lane 3) or in monolayers (lane 4), or 7E cells grown in monolayers (lane 5); 6, Bluescript *Hpa*II DNA fragments used as size markers. Sizes are indicated in bases. (B) Schematics of the E6,E7,E1^E4,E5 RNA and the probe used in assay. The 331-base fragment is derived from RNAs which are not spliced at the E4 splice junction, such as those encoding E2 and the entire early region. Undigested probe is larger because of the presence of plasmid sequences. The 208-base fragment is derived from RNAs spliced at the E4 splice junction (nt 3295). The abundance of these transcripts is increased in cells grown on rafts, while the abundance of unspliced messages is unchanged.

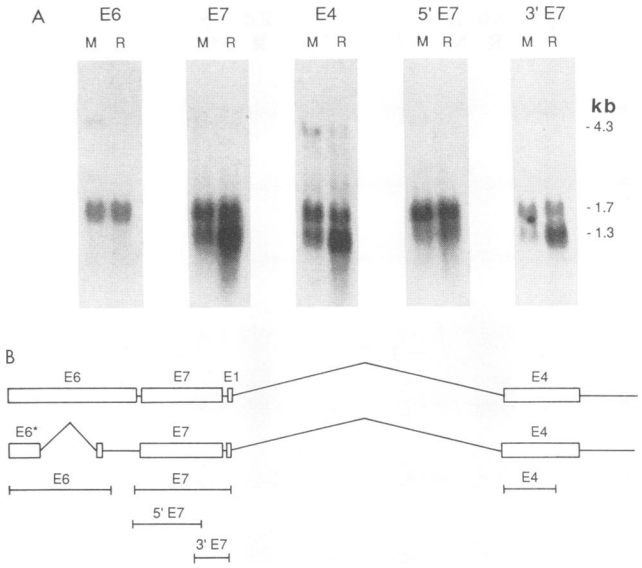

FIG. 9. (A) Mapping of the 5′ end of the differentiation-induced 1.3-kb RNA by Northern blot analysis. Probes specific for various regions of the HPV genome were generated by PCR, labeled by random priming, and hybridized under low-stringency conditions to Northern blots of total cell RNA from 9E cells grown in raft (lanes R) or monolayer (lanes M) cultures. (B) Schematics of the 1.7-kb E6*,E7,E1^E4,E5 and E6,E7,E1^E4,E5 RNAs and locations of the probes.

could be encoded by an exon upstream of E4 and joined to the E4 ORF by splicing. To confirm that the 1.3-kb transcript did not arise from an unspliced RNA initiated upstream of the E4 ORF, we performed RNase protection experiments with a probe spanning the E4 splice junction (Fig. 8). While we observed an increase in the abundance the 208-base fragment derived from transcripts spliced at the E4 splice junction in raft cultures, there was no change in the abundance of the 331-base fragment derived from unspliced messages (compare lanes 3 and 4). Thus, the 1.3-kb E4/E5 RNA induced by keratinocyte differentiation contains an exon encoded somewhere further upstream in the HPV genome which is spliced onto the E4 ORF.

Since abundant transcripts initiated in the E7 ORF and encoding the E1^E4 gene product have been previously reported in xenografts of HPV11-infected condylomas (14), we examined whether the differentiation-induced E4/E5 RNA might be initiated at a promoter within E7. To roughly map a putative small exon for the E1^E4 transcript, we first used Northern analysis with reduced-stringency hybridization conditions. In agreement with the previous high-stringency hybridization with E6/E7 probe (Fig. 2 and 6), a probe specific for the E6 ORF (nt 108 to 462) hybridized only to the 1.7-kb RNA (Fig. 9). In contrast, an E7-specific probe (nt 541 to 877) hybridized to both the 1.7- and 1.3-kb RNAs. Further dissection of the E7 ORF showed that while the 5′ 239 bases of E7 (nt 541 to 780) hybridized weakly to the 1.3-kb RNA, a 114-bp probe containing the 3′ end of E7 hybridized strongly to a 1.3-kb RNA induced by differentiation. This result indicates that the 5′ end of this transcript is located near the 3′ end of the E7 ORF. RNase protection analysis with a probe which contained 193 bases upstream and 129 bases downstream of the E1 splice junction revealed a 130- to 135-base protected fragment which was induced in raft cultures (Fig. 10). This fragment is the size expected from a transcript initiated at ~nt 742 and spliced at the E1 splice junction at nt 877 or from an RNA containing an exon encoded by nt 742 to 877. In addition, we observed a protected fragment of 193 bases, which would be expected from the 1.7-kb RNAs encoding E6/E6* and E7 and spliced at the E1 splice junction, and a fragment of 322 bases derived from unspliced transcripts such as the 4.3-kb RNA (Fig. 2). Because of incomplete digestion with RNase, some full-length probe is also present in this experiment. To confirm that the 1.3-kb RNA initiated at ~nt 742, primer extension analysis using a primer from E4 was performed. Extension products consistent with a differentiation-induced transcript initiated at ~nt 742 were observed (data not shown). We therefore conclude that the promoter for the 1.3-kb E1^E4,E5 RNA is likely to be at nt 742.

To detect additional E4/E5 transcripts initiated elsewhere in the genome, we performed an RNase protection assay with a cDNA probe (Fig. 11) containing 193 bases of sequence upstream of the E1 splice junction joined to 208 bases of the E4 ORF. We observed a 401-base product derived from the E6*/E6,E7,E1^E4,E5 transcripts which

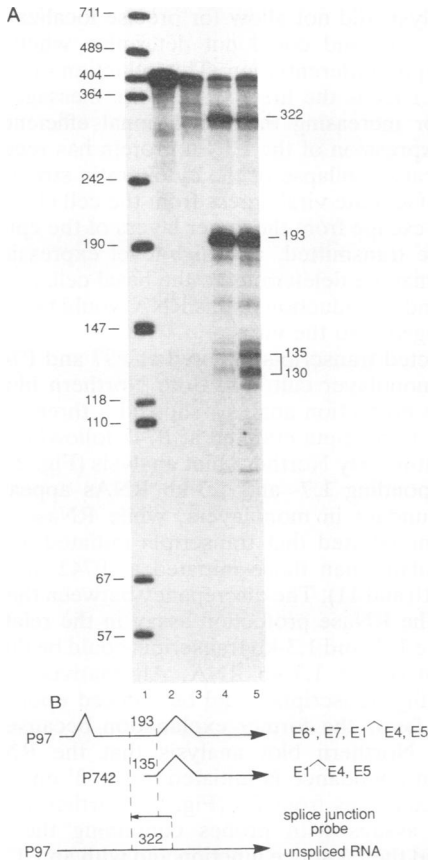

FIG. 10. (A) RNase protection analysis of RNA from raft and monolayer cultures with a probe spanning the E1 splice junction. Lanes: 1, *Hpa*II DNA fragments of Bluescript; 2, undigested probe; 3 to 5, products protected from RNase digestion by 10 μg of tRNA (lane 3) or by 10 μg of total cell RNA from 9E cells grown in monolayers (lane 4) or on rafts (lane 5). (B) Schematics of the splice junction probe and transcripts encoding E6*,E7,E1^E4,E5 initiated at P97 and the differentiation-induced E1^E4,E5 transcript initiated at P742. Sizes (in bases) of the protected regions are shown. E6,E7,E1^E4,E5 RNA would protect a product of the same size as would the E6*,E7,E1^E4,E5 RNA. The drawing is not to scale.

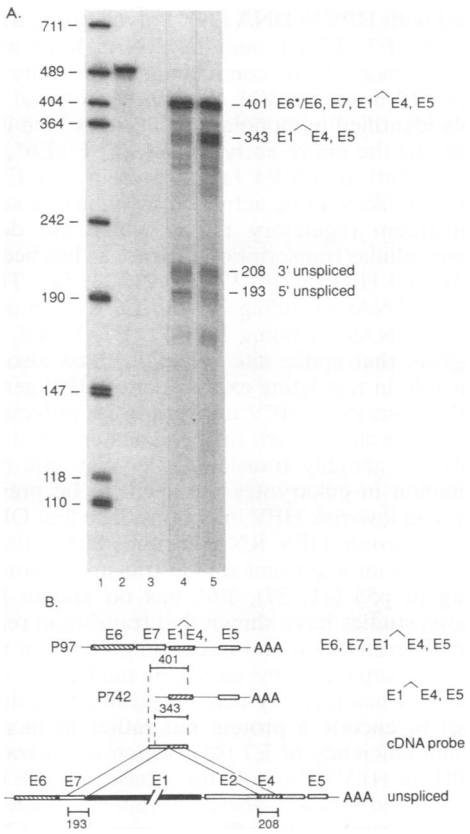

FIG. 11. (A) RNase protection assay with an E1^E4 cDNA probe. Lanes: 1, size markers; 2, undigested probe; 3, products protected by 10 μg of tRNA; 4 and 5, products protected by 10 μg of 9E total cell RNA from monolayer and raft cultures, respectively. (B) Schematics showing HPV transcripts and fragments protected by a cDNA probe. E6*,E7,E1^E4,E5 RNA would also protect a 401-base product. E6,E7,E1^E4,E5 and unspliced transcripts are initiated at nt 97 (P97); E1^E4,E5 RNA is initiated at nt 742 (P742). Boxes indicate ORFs. Sizes are indicated in bases.

was equally abundant in RNAs from rafts and monolayers. In addition, a 343-base product was found to be more abundant in raft cultures. This product is consistent in size with an E1^E4 transcript initiated at nt 742, 135 bases upstream of the E1 splice junction at nt 877. We also observed protected fragments of 208 and 193 bases, which were protected by noncontiguous portions of the 4.3-kb unspliced RNA which were present in the cDNA probe. Differentiation-induced transcripts initiated elsewhere in the HPV genome and spliced to the E4 ORF should have protected the E4-specific portion of the cDNA probe, resulting in an increase in the abundance of the 208-base fragment. No such increase was observed. Thus, the only detectable differentiation-induced transcripts encoding E4 were initiated at ~nt 742 in the E7 ORF. A Betascope analysis of the fragments protected in the cDNA RNase protection assay revealed a fourfold induction of the 343-base E1^E4,E5-protected fragment in raft RNA relative to the 401-base E6*/E6,E7,E1^E4,E5-protected fragment. This was similar to the fold increase seen in a Betascope analysis of Northern blots of raft and monolayer RNA hybridized with E4/E5 probe. Together, these data demonstrate that transcripts encoding an E1^E4 protein are initiated at ~nt 742 and are induced during keratinocyte differentiation in vitro.

DISCUSSION

We have characterized the pattern of expression of a high-risk HPV type by using an in vitro system for epithelial stratification and differentiation (rafts) which duplicates most aspects of the productive viral life cycle (34, 38). By using a cell line (CIN612) which maintains episomal copies of HPV31b (5) and allowing it to stratify in raft cultures, we have been able to identify constitutive and differentiation-activated viral promoters which most likely play important roles in the production of virus. Recently, this cell line has been used to induce the synthesis of HPV 31b virions upon differentiation in raft cultures (35a). We have used monolayer cultures as a model for basal cells and have identified polycistronic transcripts encoding E6 or E6*, E7, E1^E4, and E5 which initiate at promoter sequences, designated P97, immediately upstream of the E6 ORF. Similar messages have been observed in monolayer cultures of W12 cell lines, which contain episomal copies of HPV16 (18), and in cells

transfected with HPV16 DNA (39). Polycistronic transcripts encoding E6, E7, E1^E4, and E5 RNAs have also been observed in xenografts of condylomas induced by the low-risk types HPV6 and HPV11 (14, 40). Additional HPV31b transcripts identified in monolayer cultures which initiate at P97 encode (i) the entire early region, (ii) E6/E6*, E7, E2, and E5, and (iii) an E6^E4 fusion protein and E5. These transcripts are likely to be activated by enhancer sequences in the upstream regulatory region which are dependent solely upon cellular transcription factors, as has been shown for HPV16 and HPV18 (10, 12, 15, 23, 33, 54). The abundance of the RNAs encoding E2 and E6^E4 is much lower than that of RNAs encoding E6/E6*, E7, E1^E4, and E5. This suggests that splice site selection must also play an important role in regulating expression of HPV genes.

Since the majority of HPV transcripts are polycistronic in nature, it is not clear which of the genes encoded by these transcripts are actually translated. Polycistronic messages are uncommon in eukaryotes yet seem to be prevalent in both high- and low-risk HPV infections. The first ORF in the 1.7-kb polycistronic HPV RNA encodes either E6 or E6*. While E6 plays an important role in transformation through its binding of p53 (41, 57), E6* has no known function. Mutagenesis studies have shown that translation reinitiation can occur if termination and initiation codons on the same transcript are separated by sufficient nucleotide sequence (31). In fact, it has recently been suggested that the role of E6* is not to encode a protein but rather to increase the translational efficiency of E7 (45), which is separated from the E6 ORF in HPV31 by only three bases. The E7-specific promoter utilized by the low-risk strains may have evolved as an alternate strategy for efficient expression of E7, since these strains lack the splice sites required to make E6*. Whether E1^E4 and E5, the third and fourth genes encoded by the 1.7-kb RNA, are actually translated from this message is currently under study, using HPV31b-specific antibodies.

Transcripts which initiate at the P97 promoter are expressed in basal cells as well as throughout the stratifying epithelium. There is no apparent change in the level of transcripts which initiate at this promoter in raft cultures. In contrast, a striking increase in the abundance of a 1.3-kb RNA encoding E4 and E5 was observed by Northern blot analysis of RNA isolated from our in vitro raft system (Fig. 6). This result is consistent with those of in situ hybridization studies on HPV16-positive biopsy material from a CIN I lesion (16). RNase protection assays with a probe spanning the E4 splice junction showed a differentiation-dependent increase in the abundance of a transcript spliced at the E4 splice site (Fig. 8). In this analysis, the protected fragment is derived from both the 1.3-kb RNA and the abundant 1.7-kb RNA, which is not induced by differentiation. The presence of the constitutively expressed 1.7-kb RNA obscures the dramatic induction of the 1.3-kb RNA that is seen in Northern analysis and in other protection assays (Fig. 10 and 11). Three independent analyses using Northern blots (Fig. 9), RNase protection (Fig. 10 and 11), and primer extension demonstrated that the 1.3-kb E4/E5 RNA initiates at a promoter in the E7 gene (P742). While we cannot exclude the possibility that there is a very small 5′ exon derived from upstream sequences, we feel this is unlikely, since the sequence near P742 does not match that of a consensus splice acceptor site. We therefore believe that the initiation site of the differentiation-induced E1^E4,E5 transcript is at nt 742. Furthermore, this initiation site is similar to that of an abundant E1^E4,E5 RNA identified by R looping in an HPV11-infected condyloma (14). However, electron microscopic analysis did not allow for precise localization of the RNA start site and could not determine whether it was activated upon differentiation. The induction of a transcript in which E1^E4 is the first ORF on the message may be a strategy for increasing the translational efficiency of this protein. Expression of the E1^E4 protein has recently been shown to cause collapse of the cytokeratin structure and is thought to facilitate viral egress from the cell (17). Since the virus must escape from the upper layers of the epithelium in order to be transmitted, and high-level expression of this transcript may be deleterious to the basal cells, a differentiation-dependent induction of this RNA would be expected to be advantageous to the virus.

We detected transcripts initiated at P97 and P742 in both rafts and monolayer cultures. Both Northern blot analysis and RNase protection analysis showed a three- to fourfold increase in transcripts initiated at P742 following differentiation in culture. By Northern blot analysis (Fig. 2, 6, and 9), the corresponding 1.7- and 1.3-kb RNAs appeared to be equally abundant in monolayers, while RNase protection assays demonstrated that transcripts initiated at P97 were more abundant than those initiated at P742 in monolayer cells (Fig. 10 and 11). The discrepancy between the Northern blots and the RNase protection assay in the relative abundance of the 1.7- and 1.3-kb transcripts could be due to slight degradation of the 1.7-kb RNA. Alternatively, additional E4-containing transcripts could be induced upon differentiation. We favor the former explanation because we have shown by Northern blot analysis that the RNA which increases in abundance is initiated at the 3′ end of E7 and contains sequences from E4 (Fig. 9). Furthermore, RNase protection assays with probes containing the E4 splice junction and the E1 splice junction and with an E1^E4 cDNA demonstrated that the only sequences which increase in abundance upon differentiation are those which are present in the E1^E4 RNA.

We also observed a low-level induction of transcripts from the late region which are capable of encoding L1 and L2 proteins. Experiments are in progress to sequence these transcripts and to map their initiation sites. In the low-risk HPV6 and HPV11 strains, late-region transcripts use the same initiation site in E7 as does the E1^E4,E5 RNA (14). It will be interesting to determine whether this is also the case with HPV31b. If so, selection of splicing and polyadenylation sites must be an important feature in regulating expression of these genes.

Examination of the sequences immediately upstream of the P742 initiation site do not reveal any TATA-like sequences. However, surrounding the initiation site (nt 741 to 748) is the sequence GTGAGCTC. This sequence matches 6 of 8 nt of the CTCANCTC initiator element sequence which directs initiation of the TATA-less terminal transferase gene (47) and may be required for initiation of the E1^E4 transcript. Many genes lacking TATA sequences are regulated during development or differentiation (46). It is also interesting to note that a CCAAT sequence occurs 35 bases upstream of P742. This may be a binding site for the CCAAT enhancer-binding protein which promotes terminal differentiation of adipocytes and may also be important in differentiation of other tissues (55). This protein is expressed in differentiated keratinocytes but not in basal cells and may be involved in induction of the E1^E4,E5 HPV transcript. Also of note are the E2 binding sites located adjacent to the P97 sequences, which are approximately 650 bp away from P742. While a role for E2 as a negative regulator of P97-directed expression has been proposed, no role for E2 as a positive

transcriptional regulator has yet been identified in the high-risk HPV types. The effect of E2 on P742 deserves further investigation. Identification of the *cis*- and *trans*-acting factors required for the differentiation-dependent induction of HPV transcripts should be possible with use of the raft system and will provide further insight into the mechanisms by which the HPV life cycle is regulated in stratifying epithelium.

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REFERENCES

1. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. Nature (London) **325**:70–73.
2. **Assinleau, D., and M. Prunieras.** 1984. Reconstruction of simplified skin-control of fabrication. Br. J. Dermatol. Suppl. **111**:219–221.
3. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
4. **Barbosa, M. S., and R. Schlegel.** 1989. The E6 and E7 genes of HPV-18 are sufficient for inducing two-stage in vitro transformation of human keratinocytes. Oncogene **4**:1529–1532.
5. **Bedell, M. A., J. B. Hudson, T. R. Golub, M. E. Turek, M. Hosken, G. D. Wilbanks, and L. A. Laimins.** 1991. Amplification of human papillomavirus genomes in vitro is dependent on epithelial differentiation. J. Virol. **65**:2254–2260.
6. **Bedell, M. A., K. H. Jones, S. R. Grossman, and L. A. Laimins.** 1989. Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. J. Virol. **63**:1247–1255.
7. **Bell, G. I., J. H. Karam, and W. J. Rutter.** 1981. Polymorphic DNA region adjacent to the 5′ end of the insulin gene. Proc. Natl. Acad. Sci. USA **78**:5759–5763.
8. **Boshart, M., L. Gissmann, H. Ikenberg, A. Kleinheinz, W. Scheurlen, and H. zur Hausen.** 1984. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. EMBO J. **3**:1151–1157.
9. **Broker, T. R.** 1987. Structure and genetic expression of papillomaviruses. Obstet. Gynecol. Clin. North Am. **14**:329–348.
10. **Chin, M. T., R. Hirochika, H. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. J. Virol. **62**:2994–3002.
11. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
12. **Chong, T., W.-K. Chan, and H.-U. Bernard.** 1990. Transcriptional activation of human papillomavirus 16 by nuclear factor 1, AP1, steroid receptors and a possibly novel transcription factor, PVF: a model for the composition of genital papillomavirus enhancers. Nucleic Acids Res. **18**:465–470.
13. **Choo, K.-B., W.-F. Cheung, L.-N. Liew, H.-H. Lee, and S. H. Han.** 1989. Presence of catenated human papillomavirus type 16 episomes in a cervical carcinoma cell line. J. Virol. **63**:782–789.
14. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61**:2581–2588.
15. **Cripe, T. P., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid III, M. Durst, L. Gissman, A. Roman, and L. Turek.** 1987. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO J. **6**:3745–3753.
16. **Crum, C. P., G. Nuovo, D. Friedman, and S. J. Silverstein.** 1988. Accumulation of RNA homologous to human papillomavirus type 16 open reading frames in genital precancers. J. Virol. **62**:84–90.

16a. **deVilliers, E.-M.** 1989. Heterogeneity of the human papillomavirus group. J. Virol. **63**:4898–4903.

17. **Doorbar, J., S. Ely, J. Sterling, C. McLean, and L. Crawford.** 1991. Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. Nature (London) **352**:824–827.
18. **Doorbar, J., A. Parton, K. Hartley, L. Banks, T. Crook, M. Stanley, and L. Crawford.** 1990. Detection of novel splicing patterns in a HPV 16-containing keratinocyte cell line. Virology **178**:254–262.
19. **Durst, M., L. Gissman, H. Ikenberg, and H. zur Hausen.** 1983. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. Proc. Natl. Acad. Sci. USA **80**:3812–3815.
20. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243**:934–936.
21. **Gilman, M.** 1989. Ribonuclease protection assay, p. 4.7.1–4.7.8. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. John Wiley & Sons, New York.
22. **Goldsborough, M. D., D. DiSilvestre, G. F. Temple, and A. T. Lorincz.** 1989. Nucleotide sequence of human papillomavirus type 31: a cervical neoplasia-associated virus. Virology **171**: 306–311.
23. **Guis, D., S. Grossman, M. A. Bedell, and L. A. Laimins.** 1988. Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J. Virol. **62**:665–672.
24. **Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J. **7**:525–531.
25. **Hudson, J. B., M. A. Bedell, D. J. McCance, and L. A. Laimins.** 1990. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. J. Virol. **64**:519–526.
26. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. Academic Press, N.Y.
27. **Kaur, P., and J. K. McDougall.** 1988. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J. Virol. **62**:1917–1924.
28. **Kingston, R. E.** 1987. Primer extension, p. 4.8.1–4.8.3. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. John Wiley & Sons, Wiley, N.Y.
29. **Kopan, R., G. Traska, and E. Fuchs.** 1987. Retinoids as important regulators of terminal differentiation: examining keratin expression in individual epidermal cells at various stages of keratinization. J. Cell Biol. **105**:427–440.
30. **Koss, L. G.** 1987. Cytologic and histologic manifestations of human papillomavirus infection of the female genital tract and their clinical significance. Cancer **60**:1942–1950.
31. **Liu, C.-C., C. C. Simonsen, and A. D. Levinson.** 1984. Initiation of translation at internal AUG codons in mammalian cells. Nature (London) **309**:82–85.
32. **Lorincz, A. T., W. D. Lancaster, and G. F. Temple.** 1986. Cloning and characterization of the DNA of a new human papillomavirus from a woman with dysplasia of the uterine cervix. J. Virol. **58**:225–229.
33. **Mack, D. H., and L. A. Laimins.** 1991. A keratinocyte-specific transcription factor, KRF-1, interacts with AP-1 to activate expression of human papillomavirus type 18 in squamous epithelial cells. Proc. Natl. Acad. Sci. USA **88**:9102–9106.
34. **McCance, D. J., R. Kopan, E. Fuchs, and L. A. Laimins.** 1988. Human papillomavirus type 16 alters human epithelial cell

differentiation in vitro. Proc. Natl. Acad. Sci. USA **85**:7169–7173.

35. **McNab, J. S., J. Walkinshaw, J. Cordiner, and J. B. Clements.** 1986. Human papillomavirus in clinically and histologically normal tissue of patients with genital cancer. N. Engl. J. Med. **315**:1052–1058.

35a. **Meyers, C., M. Frattini, J. Hudson, and L. A. Laimins.** Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. Science, in press.

36. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63**:4417–4421.

37. **Pfister, H.** 1987. Relationship of papillomaviruses to anogenital cancer. Obstet. Gynecol. Clin. North Am. **14**:349–361.

38. **Rader, J. S., T. R. Golub, J. B. Hudson, D. Patel, M. A. Bedell, and L. A. Laimins.** 1990. In vitro differentiation of epithelial cells from cervical neoplasias resembles in vivo lesions. Oncogene **5**:571–576.

39. **Rohlfs, M., S. Winkenbach, S. Meyer, T. Rupp, and M. Durst.** 1991. Viral transcription in human keratinocyte cell lines immortalized by human papillomavirus type-16. Virology **183**:331–342.

40. **Rotenberg, M. O., L. T. Chow, and T. R. Broker.** 1989. Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins, using the polymerase chain reaction. Virology **172**:489–497.

41. **Scheffner, M., B. A. Werness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63**:1129–1136.

42. **Schneider-Gadicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**:2285–2292.

43. **Schwarz, E., U. K. Freese, L. Gissman, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.

44. **Schwarz, E., A. Schneider-Gadicke, and H. zur Hausen.** 1987. Human papillomavirus type-18 transcription in cervical carcinoma cell lines and in human cell hybrids. Cancer Cells **5**:47–53.

45. **Sedman, S. A., M. S. Barbosa, W. C. Vass, N. L. Hubbert, J. A. Haas, D. R. Lowy, and J. T. Schiller.** 1991. The full-length E6 protein of human papillomavirus type 16 has transforming and *trans*-activating activities and cooperates with E7 to immortalize keratinocytes in culture. J. Virol. **65**:4860–4866.

46. **Smale, S. T., and D. Baltimore.** 1989. The "initiator" as a transcription control element. Cell **57**:103–113.

47. **Smale, S. T., M. C. Schmidt, A. J. Berk, and D. Baltimore.** 1990. Transcriptional activation by Sp1 as directed through TATA or initiator: specific requirement for mammalian transcription factor IID. Proc. Natl. Acad. Sci. USA **87**:4509–4513.

48. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63**:1441–1447.

49. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.

50. **Spalholz, B. A., Y. C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.

51. **Stoler, M. H., and T. R. Broker.** 1986. In situ hybridization detection of human papillomavirus DNA and messenger RNA in genital condylomas and a cervical carcinoma. Hum. Pathol. **17**:1250–1258.

52. **Stoler, M. H., S. M. Wolinsky, A. Whitbeck, T. R. Broker, and L. T. Chow.** 1989. Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by in situ hybridization with message-specific RNA probes. Virology **172**:331–340.

53. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the in vitro transforming activities of human papillomavirus types. EMBO J. **7**:1815–1820.

54. **Thierry, F., A. G. Carranca, and M. Yaniv.** 1987. Elements that control transcription of HPV18. Cancer Cells **5**:23–32.

55. **Umek, R. M., A. D. Friedman, and S. L. McKnight.** 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. Science **251**:288–292.

56. **Ustav, M., and A. Stenlund.** 1991. Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. EMBO J. **10**:449–457.

57. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248**:76–79.

58. **Woodworth, C. D., J. Doniger, and J. A. DiPaolo.** 1989. Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. J. Virol. **63**:159–164.

59. **zur Hausen, H.** 1985. Genital papillomavirus infections. Prog. Med. Virol. **32**:15–21.

60. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancer, p. 245–263. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae. Plenum Press, New York.